Reference: *Biol. Bull.* **205:** 16–25. (August 2003)

Behavioral Characterization of Attractin, a Water-Borne Peptide Pheromone in the Genus *Aplysia*

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Abstract. Pheromones play a significant role in coordinating reproductive activity in many animals, including opisthobranch molluscs of the genus *Aplysia*. Although solitary during most of the year, these simultaneous hermaphrodites gather into breeding aggregations during the reproductive season. The aggregations contain both mating and egg-laying animals and are associated with masses of egg cordons. The egg cordons are a source of pheromones that attract other *Aplysia* to the area, reduce their latency to mating, and induce egg laying. One of these water-borne egg cordon pheromones ("attractin") has been characterized and shown to be attractive in T-maze assays. Attractin is the first water-borne peptide pheromone characterized in invertebrates.

In the current studies, behavioral assays were used to better characterize the attraction, and to examine whether attractin can induce mating. Although the two activities could be related (*i.e.*, attraction occurring because animals were looking for a partner), this was not tested. T-maze assays showed that attractin works as part of a bouquet of odors: the peptide is attractive only when *Aplysia brasiliana* is part of the stimulus. The animal does not need to be a conspecific, perhaps explaining why multiple species may be associated with one aggregation. Native and recombinant attractin are equally attractive, verifying that *N*-glycosylation at residue 8 is not required for attraction.

Mating studies showed that both native and recombinant attractin reduce the latency to mating. The effects are larger when hermaphroditic mating is considered: in addition to reducing latency, attractin doubles the number of pairs mating as hermaphrodites. The effect may result from attractin stimulating both animals to mate as males and would be consistent with behaviors previously seen in the T-maze. Attractin may thus be contributing to the formation of copulatory chains and rings seen in aggregations in the field.

These results may be interpreted in two ways: (1) attractin has multiple activities that contribute to the establishment and maintenance of the aggregation; or (2) the induced desire to mate may make attractin attractive when it is presented in conjunction with an animal. In either case, the results open the door for cellular and molecular studies of mechanism of action.

Received 8 October 2002; accepted 16 April 2003.

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Abbreviations: ASW, artificial seawater; Att, attractin; CH_3CN, acetonitrile; HFBA, heptafluorobutyric acid; M-REP, Marine Research and Educational Products; RP-HPLC, reversed-phase high performance liquid chromatography.

Introduction

Chemical communication is the most ancient form of communication and is used by most, if not all, animals examined. The organisms include, for example, ciliated protozoans (Luporini *et al.*, 1995), yeast (Kodama *et al.*, 2003), insects (Monsma and Wolfner, 1988; Roelofs *et al.*, 2002; Saudan *et al.*, 2002), molluscs (Painter *et al.*, 1998), worms (Ram *et al.*, 1999), fish (Li *et al.*, 2002), amphibians (Kikuyama *et al.*, 1995; Rollmann *et al.*, 1999; Wabnitz *et al.*, 1999), rodents (Stowers *et al.*, 2002; Novotny, 2003) and humans (Savic *et al.*, 2001.). The number of pheromones characterized in each species depends, at least in part, on the chemical nature of the pheromones and on whether the pheromones are water-borne.

Opisthobranch molluscs of the genus *Aplysia* are simultaneous hermaphrodites that do not normally fertilize their own eggs. Field studies (Kupfermann and Carew, 1974; Audesirk, 1979; Susswein *et al.*, 1983, 1984) have shown that they are solitary animals that move into breeding ag-

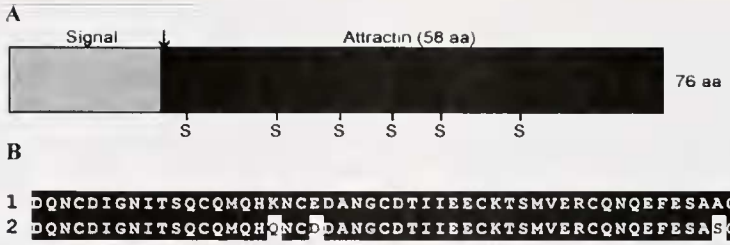

Figure 1. (A) Schematic diagram of the precursor to the attractin pheromone from the albumen gland of *Aplysia californica*. Cleavage of the signal sequence (arrow) generates the 58-residue pheromone attractin. The disulfide-bonding pattern of cysteine residues (S) is I–IV, II–V, and III–IV, where the Roman numeral indicates the order of occurrence in the primary sequence (Schein *et al.*, 2001). Unlike attractin, the precursors for pheromones that act as part of a group of scents often contain sequences of more than one scent. (B) The amino acid sequences of attractin from the two species of *Aplysia* used in the current studies, *A. californica* and *A. brasiliana* (Painter *et al.*, 1998, 2000). Amino acid residues that are identical to those in *A. californica* attractin are indicated by the black background.

gregations during the reproductive season. The aggregations typically contain both mating and egg-laying animals and are associated with masses of recently deposited egg cordons, often deposited one on top of another. Most of the egg-laying animals mate simultaneously as females even though mating does not cause reflex ovulation (Blankenship *et al.*, 1983), suggesting that egg laying precedes mating in the aggregation and that egg laying may release pheromones that establish and maintain the aggregation.

Similar observations have been made in the laboratory when animals were not individually caged (Audesirk, 1979; Blankenship *et al.*, 1983; Susswein *et al.*, 1983, 1984), and behavioral studies have shown that egg-laying animals with cordons are more attractive than sexually mature but non-laying conspecifics (Aspey and Blankenship, 1976; Jahan-Parwar, 1976; Audesirk, 1977; Painter *et al.*, 1989). T-maze assays show that at least some of the attractants derive from the egg cordon and are waterborne: (1) recent egg-layers without egg cordons are no more attractive than non-laying conspecifics; (2) recently deposited egg cordons are attractive, with or without a non-laying conspecific, but sham egg cordons are not; and (3) both recently deposited egg cordons and their eluates increase the attractiveness of non-laying conspecifics when placed in the surrounding seawater (Painter *et al.*, 1991; Painter, 1992).

One of the water-borne pheromonal attractants has been isolated from eluates of the egg cordon and characterized. Named attractin, it is a 58-residue peptide that has six cysteines that form three intramolecular disulfide bonds (Fig. 1; Painter *et al.*, 1998; Schein *et al.*, 2001). Attractin was isolated from a Pacific Coast species (*A. californica*) and bioassayed in a species from the Gulf of Mexico (*A. brasiliana*). This was done because individuals of *A. californica* tend to crawl out of T-maze chambers before they are exposed to the stimulus. *A. californica* attractin was attractive to *A. brasiliana* and produced behaviors that were suggestive of mating (Painter *et al.*, 1998), but these behaviors were not further analyzed. The amount of attractin that was attractive to conspecifics and induced the potential mating behaviors (1–10 pmol in 6 l artificial seawater) was in the range of concentrations normally observed with pheromones, demonstrating that attractin has pheromonal activity.

There is no geographical overlap between the distributions of the two species, suggesting that attractin or an attractin-related peptide is a pheromonal attractant in *A. brasiliana*. A peptide was subsequently isolated from the *A. brasiliana* albumen gland and sequenced. It is 58 amino acids in length and differs from *A. californica* attractin at only 3 amino acids (Fig. 1; Painter *et al.*, 2000). It is deposited on the egg cordon and elutes into the seawater following deposition. It could thus serve a pheromonal function in *A. brasiliana*, but its pheromonal activities have yet to be tested.

In the present study, behavioral assays were used to better characterize the attraction and to examine whether mating is induced. The current T-maze assays showed that attractin works as part of a bouquet of water-borne odors: the peptide is attractive only when individuals of *A. brasiliana* or *A. californica* are part of the stimulus. The animal does not need to be a conspecific, perhaps explaining why multiple species of *Aplysia* may be associated with one aggregation—for example, *A. vaccaria* with *A. californica* aggregations (Kupfermann and Carew, 1974; Pennings, 1991); *A. californica* with *A. vaccaria* aggregations (S. LePage, Marine Research and Educational Products (M-REP), pers. comm.); and *A. depilans* with *A. fasciata* aggregations (Achituv and Susswein, 1985). Recombinant attractin was also tested for two reasons: (1) to see whether *N*-glycosylation at Asn^8 is necessary for attraction (native attractin is glycosylated and recombinant attractin is not); and (2) to see whether the two are equally attractive, so that recombinant attractin could be used in 3D nuclear magnetic resonance solution structure studies (Garimella *et al.*, 2003) and for future studies of mechanism of action at the receptor level.

A series of mating assays was performed because behav-

iors in earlier T-maze assays suggested that both the stimulus and test animals wanted to mate (Painter *et al.*, 1998). The assays showed that attractin reduces the latency to mating at concentrations consistent with a pheromone. Attractin also reduces the latency to hermaphroditic mating and doubles the number of pairs mating as hermaphrodites. This effect may result from attractin stimulating both animals to mate as males and would be consistent with behaviors seen in earlier T-maze assays (Painter *et al.*, 1998). These results suggest that attractin, acting in an aggregation where there are more animals, could be at least partially responsible for the copulatory chains and rings that have been observed. Recombinant attractin also induces mating, both one-way and as a hermaphrodite, showing that *N*-glycosylation is not required for the induction of either type of mating. The attraction and mating data demonstrate that attractin may contribute to the establishment and maintenance of breeding aggregations, and to successful reproduction.

Materials, Methods, and Results

Animals

Specimens of *Aplysia brasiliana* (Rang), ranging in weight from 100 to 500 g, were collected from South Padre Island, Texas, and were used in experiments between June and September. *A. brasiliana* was used as the experimental animal in the T-maze and mating experiments because it is more reproductively active than *A. californica* (see fig. 2 in Painter *et al.*, 1998), does not crawl out of T-mazes, makes fewer false choices, and can be collected in large numbers from the south Texas coast during the reproductive season. Previous T-maze assays (Painter *et al.*, 1998) showed that an individual of *A. brasiliana* is attracted to a non-laying conspecific and displays behaviors suggestive of mating when 10 pmol of attractin is placed in the adjacent artificial seawater, even though attractin is a product of the *A. californica* albumen gland.

The animals were housed in individual plastic cages in one of five aquaria containing recirculating artificial seawater (ASW; Instant Ocean Marine Salt, Longhorn Pet Supply, Houston, Texas). Water was maintained at room temperature (20° ± 2°C); the salinity ranged from 30 to 32 ppt. A 14:10 light:dark cycle was maintained in the aquarium facility, with the light period starting at 0600. Animals were fed dried laver in the late afternoon (1600–1800) after experiments were completed.

Egg-laying activity was checked twice every day (0800–0900, 1600–1800), egg-laying activity was recorded, and egg cordons were removed. All animals used in assays were sexually mature, as defined by the ability to lay eggs spontaneously or in response to injection of atrial gland extracts (made as described in Painter *et al.*, 1991).

Specimens of *A. californica* (Cooper) were obtained from Alacrity Marine Biological Services (Redondo Beach, California) and M-REP (Escondido, California). They were maintained as described above, except that the water temperature was 14° ± 2°C. This species was used as a stimulus animal in one set of T-maze assays and as the source of albumen glands for purification of native attractin.

Purification of native and recombinant attractin

Procedures. Attractin from the albumen gland of *A. californica* was purified by analytical C18 reversed-phase high performance liquid chromatography (RP-HPLC) as previously described (Painter *et al.*, 1998). To prepare recombinant attractin, the *A. californica* albumen-gland attractin cDNA (Fan *et al.*, 1997) was subcloned into the baculovirus expression vector pFastBac 1, and recombinant virus was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Attractin was expressed in Sf9 insect cells grown at 27–28 °C in Sf-900 II serum-free medium (Invitrogen).

Expressing Sf9 cells were centrifuged, and the pellet was resuspended in 20 ml of ice-cold 0.1% heptafluorobutyric acid (HFBA) and sonicated. The resulting lysates were purified on C18 Sep-Pak Vac cartridges (5 g; Waters Corp.) that were pretreated with 10 ml of 100% acetonitrile (CH_3CN) containing 0.1% HFBA and rinsed with 20 ml of 0.1% HFBA. The peptides were loaded, eluted with 15 ml of 50% CH_3CN containing 0.1% HFBA, and lyophilized. The lyophilizate was resuspended in 2.5 ml of 0.1% HFBA and applied to a Vydac analytical C18 RP-HPLC column (4.6 × 250 mm).

The column was eluted with a two-step linear gradient of 0.1% HFBA in water and 100% CH_3CN containing 0.1% HFBA. The first step was 0%–10% CH_3CN in 5 min, followed by a shallower gradient from 10% to 34% CH_3CN in 85 min. The column eluate was monitored at 215 nm, and 1-min (1 ml) fractions were collected. The attractin-containing fractions were combined, lyophilized, and repurified by Vydac C18 RP-HPLC. The same gradient conditions were used as described above, except that 0.1% trifluoroacetic acid was the counterion.

Results. The RP-HPLC peak fractions containing *A. californica* recombinant attractin, identified by comparison to the elution time of native attractin, were characterized by amino acid compositional and microsequence analyses; the 58-residue peptide sequence was identical to *A. californica* albumen-gland attractin except that, according to matrix-assisted laser desorption/ionization mass spectrometry, the native peptide is *N*-glycosylated at Asn^8 and the recombinant peptide is not.

Pheromonal attraction

Procedures. The T-maze, and its associated cages, is illustrated in Figure 2. Before each assay, 6 l of ASW was

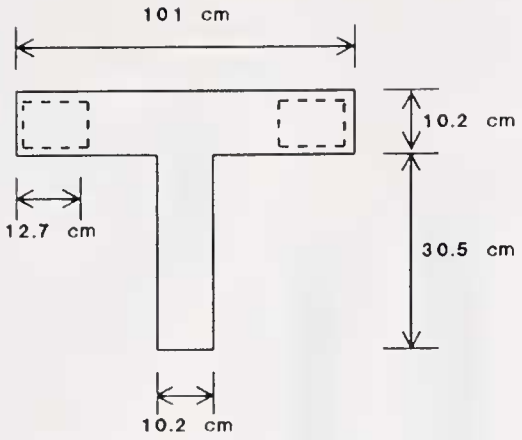

Figure 2. Schematic diagram of the T-maze with removable stimulus cages (dashed outlines) in place. T-maze depth: 10.2 cm.

put into the maze; the ASW was stationary during experiments. To minimize the amount of stress experienced by the animals during transfer to the maze, the ASW was similar in temperature and salinity to that in the aquarium from which the animals were taken. The ASW placed in the maze had not previously contacted *A. californica* or *A. brasiliana*, because there are animal-derived factors that make a non-laying conspecific attractive (Painter *et al.*, 1991).

A non-laying conspecific was placed in one of the stimulus cages and a potential attractant added to the adjacent ASW; this is the stimulus animal. After 5 min, a non-laying animal, known as the test animal, was placed in the base of the maze and watched for up to 20 min. In most cases, the test animal moved directly to the top of the maze and exhibited one of two patterns of behavior. (1) It stopped, moved its head from side to side, then either moved into one arm or returned to the base of the maze and remained there. (2) It swam around in the maze, often visiting both cages before deciding where to stop. A response was considered to be positive if the test animal traveled to the stimulus within 20 min, and then maintained contact with the stimulus cage for 5 min. It was negative if the test animal traveled to the cage in the opposite arm and maintained contact for 5 min. The response was considered to be no choice if the test animal did neither. Ten assays were performed for every potential attractant, and the attractant was alternated between arms in consecutive assays. Statistical significance was assessed by chi-square analysis. In each case, test animals were choosing between a stimulus in one arm and no stimulus in the other.

Animals for each assay were selected on the basis of three criteria. First, the animals must have been sexually mature but not have laid eggs or been used in a bioassay during the preceding 24 h. Second, the test animal must not have been exposed previously to the fraction being tested. Third, stimulus and test animals must have been housed in the same aquarium (Painter *et al.*, 1998). An exception was made to the third criterion in one set of assays, when *A. californica* was used as the stimulus animal. *A. brasiliana* was always used as the test animal (Painter *et al.*, 1998).

Several series of experiments were performed. The first compared the attractiveness of a non-laying specimen of *A. brasiliana* with 1 pmol of either native or recombinant attractin in the adjacent ASW to a non-laying conspecific with nothing added. We were asking several questions. Are smaller amounts of native attractin attractive? Is recombinant attractin as attractive? Would it be feasible to use recombinant attractin in future behavioral, molecular, or 3D structural studies? The second series examined whether a non-laying conspecific was needed for 1 pmol attractin to be attractive. We were asking: does attractin function alone or as part of a "bouquet of scents," as other pheromones do in many systems? The third series examined whether the non-laying animal must be a conspecific. We were asking: could the presence of multiple species at one breeding aggregation be due, partially or completely, to attractin? If animal-derived factors are necessary, do they differ among species?

Results. The results of the experiments comparing the attractiveness of native and recombinant attractin are shown in Figure 3. In the negative control (non-laying conspecific with nothing placed in the adjacent ASW), two animals (20%) traveled to the right arm and remained, two (20%) traveled to the left arm and remained, and six (60%) did neither. Of the four animals making a choice, only two went to the stimulus animal, one of which was in the right arm and the other of which was in the left arm of the maze. These bioassays verify that there is no directional bias in the maze and establish chance levels of attraction at two animals.

The response pattern changed when 1 pmol of either native or recombinant attractin was placed in the seawater adjacent to the stimulus animal (Fig. 3): 9 of 10 animals (90%) were attracted to recombinant attractin, and 8 of 10 animals (80%) were attracted to native attractin; in both cases, fewer animals went to the opposite arm and fewer failed to make a choice. The response patterns for each differed significantly from that for a non-laying conspecific alone [recombinant: $\chi^2(2) = 13.75$; $P < 0.005$; native: $\chi^2(2) = 10.44$, $0.05 < P < 0.1$], but did not differ significantly from each other [$\chi^2(2) = 2.1$, $0.25 < P < 0.5$].

The results of the experiment examining whether an animal is needed for attraction are shown in Figure 3. When 1 pmol recombinant attractin was placed in the seawater without a stimulus animal, 3 of 10 animals (30%) were attracted to recombinant attractin, two animals (20%) went to the opposite arm, and five animals (50%) did neither (Fig. 3). The response pattern to 1 pmol recombinant attractin alone differed significantly from that to 1 pmol recombinant attractin with an animal [$\chi^2(2) = 6.00$; $0.025 < P < 0.05$], but did not differ from that to a non-laying conspecific alone [$\chi^2(2) = 0.277$; $0.95 < P < 0.975$].

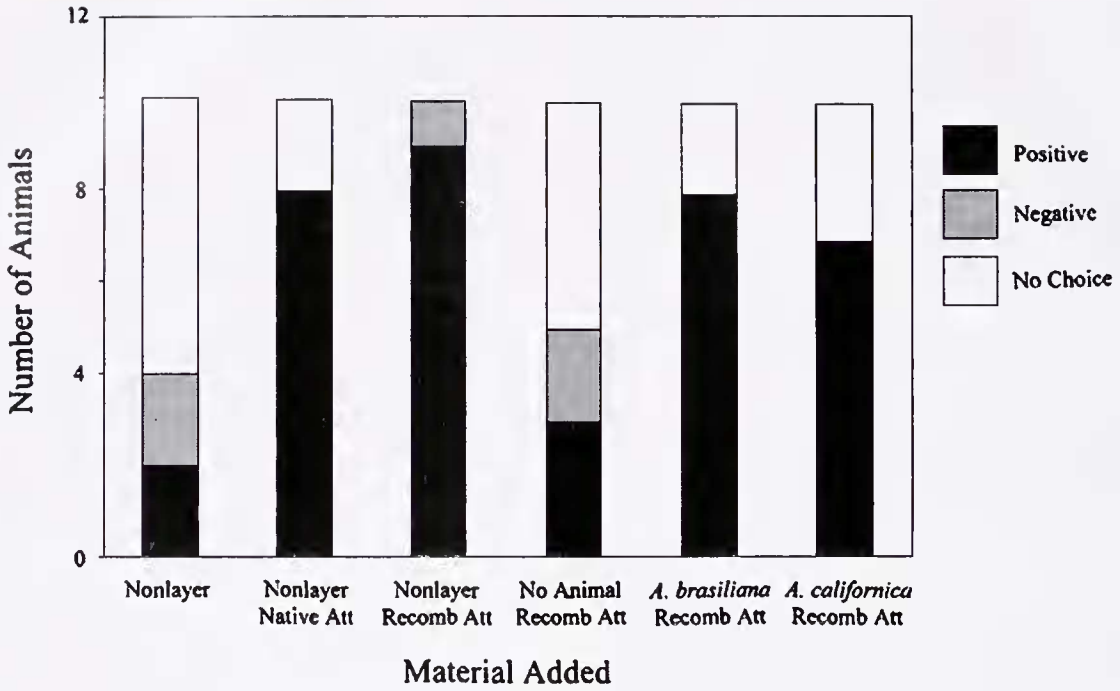

Figure 3. Both native and recombinant attractin are attractive; attractin acts in conjunction with other odors; and the animal-derived factor is not species-specific. The number of *Aplysia brasiliana* individuals attracted to a non-laying conspecific (Nonlayer) was increased by placing 1 pmol of either native attractin (Nonlayer Native Att) or recombinant attractin (Nonlayer Recomb Att) in the adjacent seawater. In each assay, animals chose between a stimulus in one arm and no stimulus in the other. Fewer *A. brasiliana* individuals were attracted to recombinant attractin when the stimulus did not contain a non-laying conspecific (No Animal Recomb Att; 1 pmol). About the same number of *A. brasiliana* individuals were attracted to the specimen of *A. californica* with recombinant attractin (*A. californica* Recomb Att; 1 pmol) as were attracted to the specimen of *A. brasiliana* with recombinant attractin (*A. brasiliana* Recomb Att; 1 pmol).

Animals do not release attractin unless they are laying eggs; therefore, the combined odor of a non-laying animal and attractin produces a qualitatively different stimulus from attractin alone. The data confirm that attractin functions as part of a bouquet of scents and led us to ask, Does the animal-derived pheromone have to come from a conspecific or can it come from a different species of *Aplysia*, perhaps accounting for the presence of multiple species at an aggregation? This would also be consistent with reports of multiple species showing up at one aggregation in the field.

The results of experiments examining whether the stimulus animal needs to be a conspecific in order for attractin to be attractive are shown in Figure 3. When 1 pmol of recombinant attractin was placed in the seawater adjacent to *A. brasiliana*, 8 of 10 *A. brasiliana* (80%) were attracted to the non-laying conspecific. When 1 pmol of recombinant attractin was placed in the seawater adjacent to *A. californica*, 7 of 10 *A. brasiliana* (70%) were attracted to the non-laying *A. californica*. The response patterns for the two species did not differ significantly from each other [$\chi^2(2) = 0.265$; $0.75 < P < 0.9$], but each differed significantly from that for a non-laying *A. brasiliana* alone [*A. brasiliana*, $\chi^2(2) = 10.44$; $0.005 < P < 0.01$; *A. californica*, $\chi^2(2) = 7.50$; $0.005 < P < 0.01$].

Pheromonal induction of mating activity

Procedures. As in the T-maze bioassays, three criteria were used to select animals for each experiment. First, the animals must have been sexually mature but not have laid eggs or been used in a bioassay during the previous 24 h. Second, the animals must not have been exposed previously to the fraction being tested or have been paired with the same animal twice. Third, both animals must have been housed in the same aquarium (Painter *et al.*, 1998).

Each assay was performed in 3 l of aerated ASW in a 4-l plastic beaker. The ASW had approximately the same osmolarity and temperature as the ASW in the aquarium from which the animals were removed, and had not previously contacted *A. brasiliana*. Animal-conditioned ASW not only increases the attractiveness of a non-laying cospecific, but also reduces the latency to mating (Painter *et al.*, 1991).

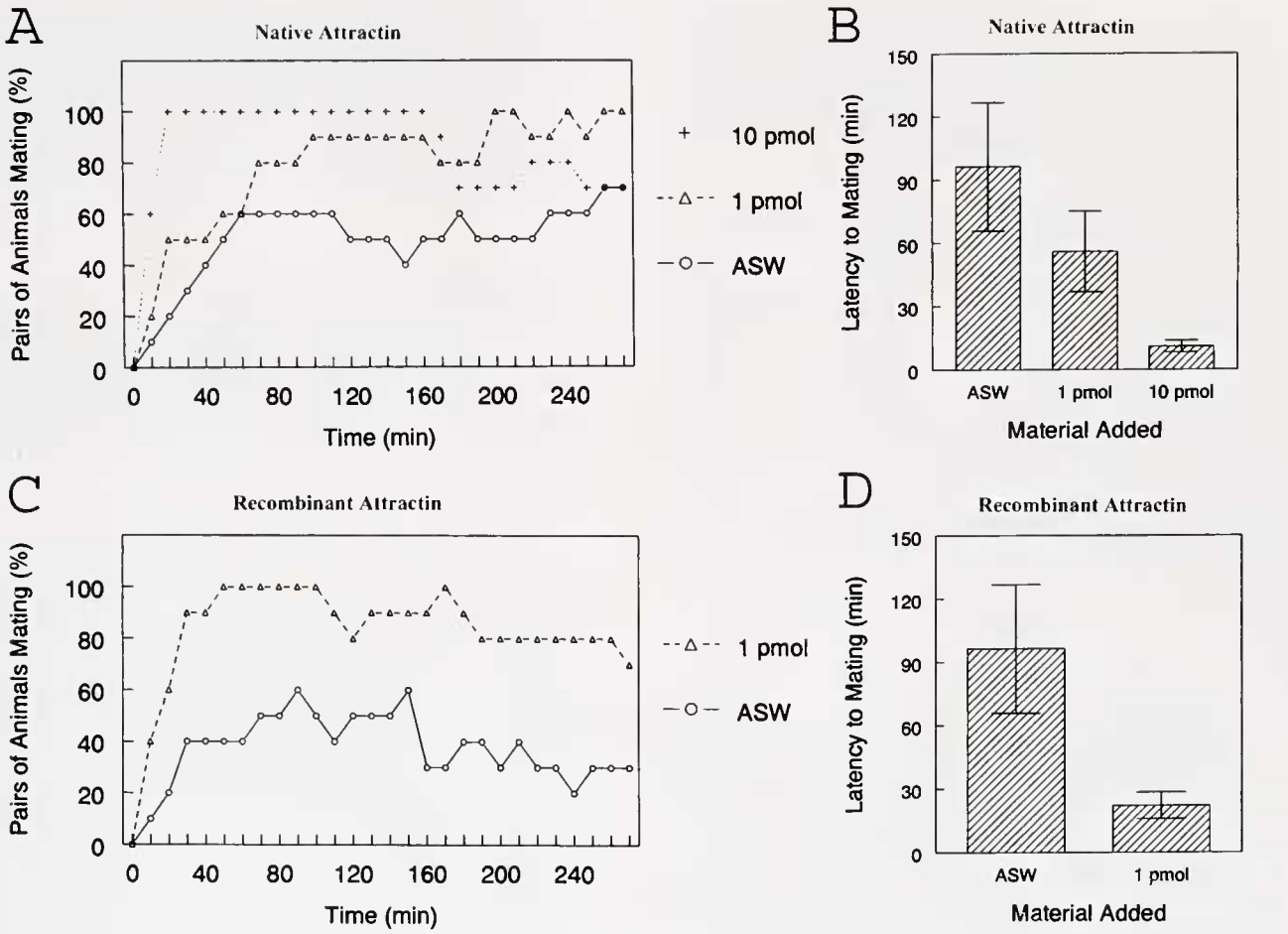

Figure 4. Both native and recombinant attractin reduce the latency to mating in *Aplysia brasiliana.* (A) The percentage of animals mating at early time periods was increased when native attractin was placed in the adjacent seawater. (B) The latency to mating was reduced by placing either 1 pmol or 10 pmol native attractin in the seawater. (C) The percentage of animals mating at early time periods was increased when recombinant attractin was placed in the adjacent seawater. (D) The latency to mating was reduced by placing 1 pmol recombinant attractin in the seawater.

Animals were rinsed in fresh non-conditioned ASW before being introduced into the experimental beaker.

Two individuals of *A. brasiliana* and a test sample were added to a beaker, and behaviors were assessed at 10-min intervals for 270 min. Three categories of behavior were identified: (1) mating as a female or male (one-way mating), (2) mating as a hermaphrodite, and (3) laying eggs. Since an egg cordon is a source of multiple contact and water-borne pheromones that modify reproductive behaviors, egg-laying activity was noted and the bioassay stopped; the bioassay for that sample was repeated with other animals. Egg laying occurred rarely with any stimulus.

Test samples included ASW with nothing added (negative control), ASW with 1 or 10 pmol native attractin added, and ASW with 1 pmol recombinant attractin added. The statistical significance of the differences between time points was determined by chi-square analysis; the statistical significance of differences in mean latency was determined by one-way analysis of variance. The same number of assays was performed for each treatment.

Results (native attractin). When 1 or 10 pmol of native attractin was placed in ASW containing two non-laying specimens of *A. brasiliana,* the percentage of animals mating at each time point (10-min intervals) was recorded. The percentage of animals mating was significantly increased for 10 pmol attractin at 10, 20, 30, and 40 min, and there was a nonsignificant trend in this direction for 1 pmol attractin (Fig. 4A). The mean latency to mating was significantly reduced for 10 pmol attractin ($\chi^2(1) > 3.84$ for each; $P < 0.05$; $n = 10$), and there was a nonsignificant trend in this direction for 1 pmol (Fig. 4B). Although the latency to mating was reduced, the overall percentage of animal pairs mating during the 270-min period was not affected (negative controls: 90% mated; native attractin: 100% mated),

perhaps reflecting the long duration of the assay or animal housing in individual cages. In these experiments, nearly all animal pairs eventually mated during the 270-min time period, regardless of whether attractin was present. Nevertheless, the results suggest that attractin facilitates, but does not induce, mating.

Results (recombinant attractin). When 1 pmol of recombinant attractin was placed in ASW containing two non-laying specimens of *A. brasiliana*, the percentage of animals mating at 10, 20, 170, and 240 min was significantly increased compared to negative controls [$\chi^2(1) > 3.84$ for each; $P < 0.05$; $n = 10$; Fig. 4C]. The mean latency to mating was significantly reduced for 1 pmol recombinant attractin ($P < 0.05$; one-way analysis of variance; Fig. 4D). Although the latency to mating was reduced, the total percentage of animal pairs that mated during the entire 270-min period was similar (negative controls: 90% mated; recombinant attractin: 100% mated), suggesting again that attractin facilitates, rather than induces, mating.

Pheromonal induction of hermaphroditic mating

Procedure. This is a re-analysis of the data collected in the mating assays, focusing on whether attractin can induce or facilitate hermaphroditic mating. As noted above, hermaphroditic mating was recorded during the mating assays.

Results (native attractin). When native attractin was placed in the ASW surrounding two non-laying specimens of *A. brasiliana*, the percentage of animal pairs mating as hermaphrodites was significantly increased for 10 pmol attractin at every time point between 20 and 170 min and for 190, 200, 230, and 250 min ($\chi^2(1) > 3.84$ for each; $P < 0.05$; $n = 10$); the same was true for 1 pmol attractin at 230, 240, and 250 min ($\chi^2(1) > 3.84$ for each; $P < 0.05$; $n = 10$) (Fig. 5A). The mean latency to hermaphroditic mating was significantly reduced for 10 pmol attractin ($P < 0.05$; one-way analysis of variance), and there was a nonsignificant trend in this direction for 1 pmol attractin (Fig. 5B). Compared to control assays, the percentage of animal pairs that mated as hermaphrodites during the 270-min period was about doubled (negative controls: 40%; 1 pmol: 70%; 10 pmol: 80%). This suggests that attractin induces, rather than facilitates, hermaphroditic mating, perhaps by stimulating both animals to mate as males. This induction could be responsible for copulatory rings and chains in the field, which may result because there are usually more than two animals in an aggregation.

Results (recombinant attractin). The percentage of animals mating as hermaphrodites at any given time point and the latency to hermaphroditic mating were not significantly increased upon addition of 1 pmol of recombinant attractin, although there were trends in this direction (Fig. 5 C, D). Although the percentage of animals mating as hermaphrodites was not significantly increased at any particular time point, the percentage of animal pairs that mated as hermaphrodites at some point during the assay did increase (negative controls: 40% mated as hermaphrodites; recombinant attractin: 70% mated as hermaphrodites). A dose of 10 pmol was not tested, which may account for the lack of statistical significance.

Discussion

We purified native attractin from extracts of *Aplysia californica* albumen gland (Painter *et al.*, 1998) and recombinant attractin from insect cells to better characterize the biological activity of the peptide and to see whether recombinant attractin could be used in future molecular studies.

Pheromonal attraction

In the T-maze, the attractiveness of a stimulus animal was significantly increased when 1 pmol of either native or recombinant attractin was placed in the adjacent seawater, verifying that both peptides are attractive in amounts consistent with pheromonal activity, and confirming that *N*-glycosylation is not required for attraction. The response patterns for the two peptides do not differ significantly from each other, demonstrating that either could be used in future studies. Recombinant attractin was therefore used in subsequent T-maze bioassays. Since it was not *N*-glycosylated, recombinant attractin was also used to determine the solution structure of the pheromone by 3D nuclear magnetic resonance (Garimella *et al.*, 2003).

Fewer individuals of *A. brasiliana* were attracted to recombinant attractin when the stimulus did not contain a non-laying conspecific, demonstrating that attractin acts in concert with other unidentified odors to stimulate attraction. These results, combined with earlier observations (the egg cordon is attractive without a stimulus animal, Painter *et al.*, 1991; attractin elutes from the egg cordon, Painter *et al.*, 1998), suggest that the composition of the bouquet of scents can vary. To identify other attractive chemical factors in the egg-cordon bouquet of scents, we have begun isolating other peptides/proteins that elute from the cordon for bioassay.

To begin looking for animal-derived attractants, we tested whether the stimulus animal needs to be a conspecific. It does not. *A. californica* with attractin and *A. brasiliana* with attractin each attracted a similar number of *A. brasiliana*. This pairing may seem inappropriate since the two species do not overlap in their geographic distributions (*A. californica*, Pacific Coast; *A. brasiliana*, Gulf of Mexico), but it may help explain why multiple *Aplysia* species are sometimes associated with one aggregation. For example, *A. californica* and *A. vaccaria* have been observed in the same breeding aggregations off the coast of California (Kupfermann and Carew, 1974; S. LePage, M-REP, pers.

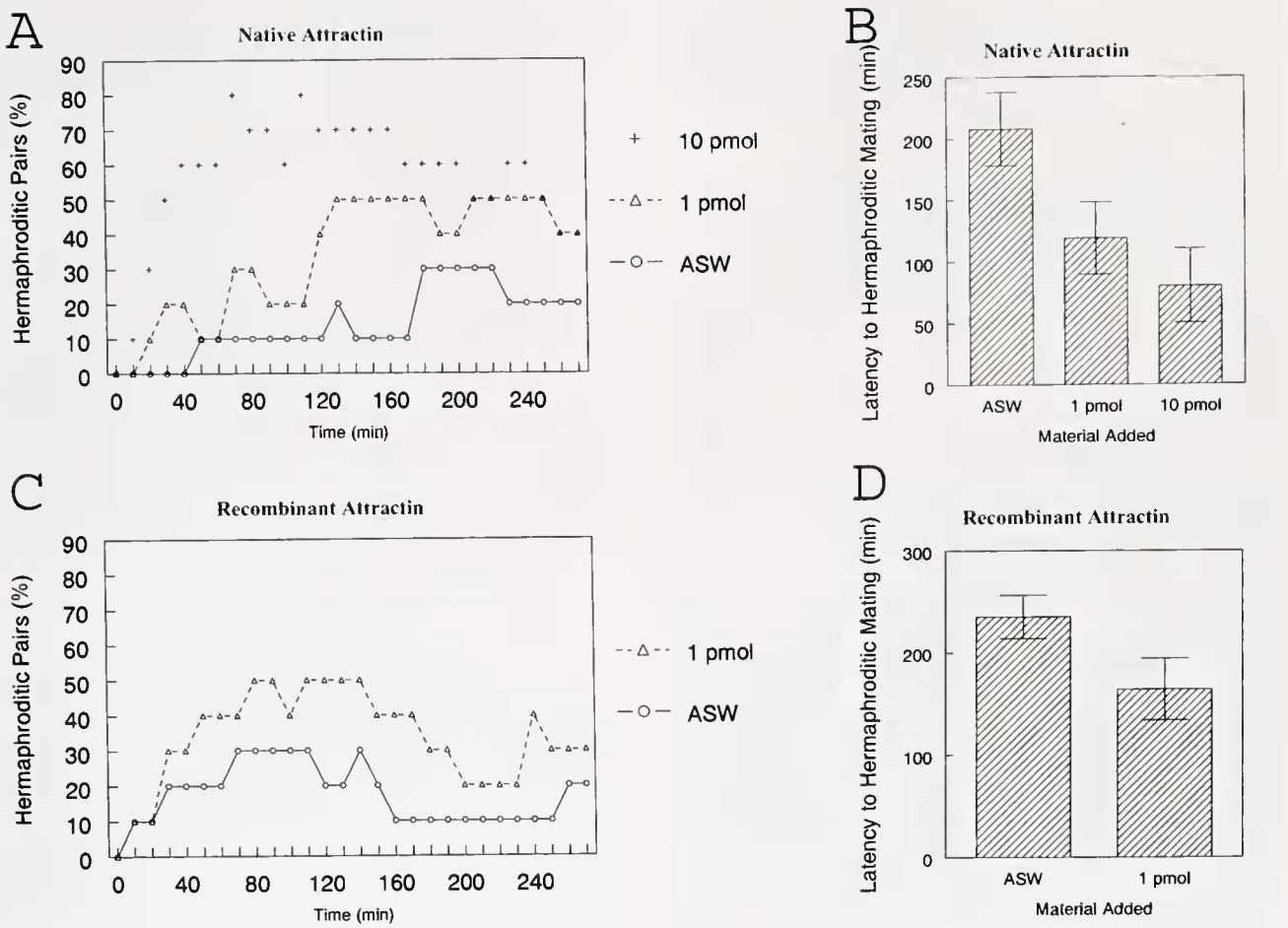

Figure 5. Both native and recombinant attractin induce hermaphroditic mating in *Aplysia brasiliana.* (A) The percentage of animals mating as hermaphrodites was increased when native attractin was placed in the adjacent seawater. (B) The latency to hermaphroditic mating was reduced by placing either 1 pmol or 10 pmol native attractin in the seawater. (C and D) The percentage of animal pairs mating as hermaphrodites was increased when 1 pmol recombinant attractin was placed in the adjacent seawater. The mean latency to hermaphroditic mating was also reduced.

comm.), and have been seen mating with each other in the aggregation (S. LePage, M-REP, pers. comm.). *A. fasciata* and *A. depilans* have also been seen associated with the same aggregation (Achituv and Susswein, 1985), but mating has not been observed because their reproductive cycles are not entirely synchronized. Audesirk (1977) previously found that *A. californica* was not attracted to conspecifics in Y-maze assays, and Audesirk and Audesirk (1977) showed that there was no seasonal effect on the sensitivity to conspecifics. Furthermore, experimental perfusion of the *A. californica* rhinophore nerve with seawater that had bathed *A. californica*, *A. vaccaria,* or *Pleurobranchia californica* evoked about the same increase in afferent activity, suggesting that aggregations of *Aplysia* species in the field are not determined by species-specific chemical cues (Chase, 1979).

Pheromonal induction of mating

Mating assays were performed because behaviors seen in earlier T-maze assays suggested that exposure to attractin could stimulate behaviors suggestive of mating as a male (Painter *et al.,* 1998). The current studies showed that when attractin is added to the seawater adjacent to a pair of *A. brasiliana,* the latency to mating is reduced relative to controls. However, the overall percentage of animal pairs mating during the prolonged assay period was not significantly different, suggesting that attractin facilitates, but does not induce, mating.

Attractin also significantly reduces the latency to hermaphroditic mating when added to the seawater surrounding a pair of *A. brasiliana.* The percentage of animal pairs mating as hermaphrodites during the assay period was about doubled, suggesting that attractin induces hermaphroditic

mating. This effect may result from attractin stimulating both animals to mate as males, as suggested by T-maze behaviors. Overall, these data suggest that attractin contributes to the establishment and maintenance of breeding aggregations.

Attractin does not stimulate species-specific attraction

The attractins appear to be a structurally diverse family of peptides, each of which is sequence-specific for a given species. Attractin has recently been characterized from *A. brasiliana*, *A. fasciata*, *A. vaccaria*, *A. depilans*, and *Bursatella leachii* and found to be 95%, 91%, 43%, 41%, and 21% identical to *A. californica* attractin, respectively (Painter *et al.*, 2000, and unpubl. data). Nevertheless, attractin is attractive to all aquatic gastropods tested to date: (1) *A. californica* attractin is attractive to *A. brasiliana* (Painter *et al.*, 1998); (2) *A. vaccaria* attractin is attractive to *A. brasiliana* (unpubl. data); and (3) *A. californica* attractin is attractive to the freshwater pulmonate *Lymnaea stagnalis* (A. ter Maat, Free University, Amsterdam, pers. comm.). Although the primary structures of attractin-related peptides are divergent, their 3D structures may be similar to *A. californica* attractin (Garimella *et al.*, 2003). To our knowledge, the attractins are the first peptide pheromone family in invertebrates that is not species-specific. In contrast, waterborne peptide pheromonal attractants in amphibians are species-specific (Kikuyama *et al.*, 2002).

There may be advantages to attracting multiple species to the same breeding aggregation. If members of a second species lay eggs on those of a different species, the mixed egg mass becomes larger, which might in some way protect the eggs of both species. Another possibility is that egg laying by one *Aplysia* species attracts a second species that then lays eggs and releases attractin, which may eventually attract members of the first species. Because attractin is continuously degraded from the C-terminus after its release (Painter *et al.*, 1998, and unpubl. obs.), it may be advantageous to attract as many individual *Aplysia* as possible, regardless of species, to lay eggs and maintain the elevated attractin concentrations needed to recruit new individuals to the breeding aggregation.

Chemical communication frequently involves the use of blends of pheromones rather than single-compound pheromones. Blends of airborne pheromones are important for species-specific signaling in many organisms, including arthropods. Mate finding in most moth species, for example, involves the release of long-distance airborne sex pheromones, which are produced in specialized female abdominal glands, generally *via* unsaturated fatty-acid precursors produced by desaturases (Roelofs *et al.*, 2002). A great diversity of pheromone structures is used throughout the Lepidoptera, even among closely related species, and the blend ratio is important for species-specific signaling. There is strong selection pressure against novel blends and response preferences (Roelofs *et al.*, 2002). Although airborne sex pheromones capable of inducing spatial orientation of conspecifics "downwind" are well established in insects (Carde and Minks, 1996), this is not the case in vertebrates, whose identified sex pheromones tend to have a small range of effectiveness; in fish, the known sex pheromones are gonadal steroids, prostaglandins, or bile acids (Li *et al.*, 2002).

Mate attraction in the genus *Aplysia*, and perhaps in other gastropods, appears to involve long-distance signaling *via* waterborne pheromone blends. Attractin by itself is not attractive to *Aplysia* species, but egg cordons alone are sufficient to attract *Aplysia* species "downstream," indicating that the cordons themselves contain a blend of pheromones. Once aggregations of multiple *Aplysia* species form, appropriate intraspecific mating may be achieved through the use of specific proximal cues involving contact chemoreception and mechanoreception (Chase, 1979).

Acknowledgments

We thank Drs. A. Susswein, A. ter Maat, and M. Miller for their interesting comments and acknowledge the University of Texas Medical Branch (UTMB) Protein Chemistry Laboratory, which is supported by the UTMB Educational Cancer Center, for compositional and microsequence analyses. This work was supported by NSF Grant IBN-9985778 (S.D.P.), and John Sealy Memorial Endowment Fund for Biomedical Research Development Grant 2579-02R (G.T.N.).

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